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CREB in the Pathophysiology of Cancer: Implications for Targeting Transcription Factors for Cancer Therapy

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Abstract

Transcription factors are key regulators of the pattern of gene expression in a cell and directly control central processes such as proliferation, survival, self-renewal, and invasion. Given this critical role, the function of transcription factors is normally regulated closely, often through transient phosphorylation. Although transcription factors are not often directly modified by mutations in cancer cells, they frequently become activated constitutively through mutations affecting “upstream” pathways. By continually driving the expression of key target genes, these oncogenic transcription factors play a central role in tumor pathogenesis. One such transcription factor is the cAMP-regulatory element-binding protein (CREB), which can be activated through phosphorylation by a number of kinases, including Akt, p90Rsk, protein kinase A, and calcium/calmodulin-dependent kinases and regulates genes whose deregulated expression promotes oncogenesis, including *cyclins*, Bcl-2 family members, and *Egr-1*. CREB is overexpressed and constitutively phosphorylated in a number of forms of human cancer, including acute myeloid leukemia (AML) and non – small cell lung cancer, and appears to play a direct role in disease pathogenesis and prognosis. Although transcription factors have not been a central focus of drug development, recent advances suggest that CREB and other such proteins may be worthwhile targets for cancer therapy.

Background

A key goal in translational cancer research is to understand the molecular abnormalities that underlie the malignant behavior of a cancer cell, and to target them specifically. Much focus in recent years has been on kinases as targets for therapy. Although dramatic advances have been made through the use of kinase inhibitors such as Bcr-Abl inhibitors in chronic myeloid

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leukemia (CML) or epidermal growth factor receptor (EGFR) kinase inhibitors in non-small cell lung cancer, a number of hurdles have arisen. First, in most forms of cancer, no single kinase emerges as being activated in a majority of cases. Even where well-defined activating events in kinases have been described, such as Her2 amplification in breast cancer, these occur in less than one-third of all patients. Second, even in tumors in which kinase inhibitors have proven beneficial, resistance to this approach develops frequently, either through mutations in the target kinase rendering them insensitive to the drug, or through activating mutations in other kinases that bypass the effects of the inhibitor.

In considering other levels of control that may play an important role in malignant cellular behavior, and which may be important targets for therapy, increasing attention has focused on transcription factors. By regulating gene expression, transcription factors are often the final regulators of such central processes as proliferation, survival, self-renewal, and invasion. Altered function or expression of a transcription factor may occur as a central component of the molecular pathogenesis of a tumor. One example is the increased expression of c-myc driven by chromosomal translocations in Burkitt's lymphoma. Alternatively, transcription factors that display normal sequence and expression may still be critical mediators of oncogenic events occurring "upstream." For example, many kinase and cell-surface receptor-triggered pathways converge on transcription factors such as NF- κ B or STAT family members. Inhibition of these transcription factors can revert the malignant behavior of many tumor types. Since normal cells are relatively insensitive to inhibition of these proteins due to redundancies in normal signaling pathways, inhibitors of these transcription factors hold the potential for having a very high therapeutic index.

Much of our initial understanding of the role of transcription factors in oncogenesis arose from studies of normal signal transduction. Growth factors and cytokines can trigger cell-cycle progression and other cellular events through the rapid and transient activation of transcription factors that regulate the expression of genes such as cyclins. The malignant phenotype in cancer is mediated by inappropriate expression of these same genes, often driven by continuous nonphysiological activation of the transcription factors that normally control their expression. Thus, critical insight into the role of transcription factor activation in cancer arose from studying the regulation of genes in response to cytokines under normal conditions. Much of the initial work on cytokine-mediated control of gene expression, proliferation, survival, and differentiation was performed in hematopoietic cells due to the relative ease in recapitulating the biology of these cells *in vitro*. Furthermore, cytokines often play a direct role in the biology of hematopoietic malignancies.

The Role of Cytokine Signaling in Leukemogenesis

Acute myeloid leukemia (AML) cells express hematopoietic growth factor receptors and have increased response to cytokines (1–4). Myeloid cell production is controlled by growth-stimulatory and -inhibitory molecules such as granulocyte macrophage-colony-stimulating factor (GM-CSF), granulocyte-colony-stimulating factor (G-CSF), and interleukin-3 (IL-3). GM-CSF and G-CSF not only stimulate the proliferation and differentiation of myeloid cells, but they also enhance granulocyte function. Both GM-CSF and G-CSF have been used to ameliorate chemotherapy- and radiation-induced neutropenia in cancer patients (5,6). Growth factors such as G-CSF and IL-3 are produced by AML cells, which, in turn, bind to their receptors and activate signaling pathways that promote cell growth and survival (7–12). Although GM-CSF and IL-3 are not required for the induction of CML-like myeloproliferative disease in mice by the BCR/ABL fusion oncoprotein, they may promote survival in premalignant cells that have yet to fully transform (13). Therefore, growth factor signaling plays an important role in both normal and neoplastic myeloid cell proliferation and survival.

The cAMP Response Element-binding Protein Links Cytokine Signaling to Nuclear Events in Myeloid Cells

Several transcription factors downstream of GM-CSF signaling regulate key target genes during myelopoiesis. *Egr-1* and *c-fos* are known target genes induced in response to GM-CSF signaling (6,14–20). CREB is a transcription factor that mediates *egr-1* transcription and is activated by GM-CSF and IL-3 (21–24). This finding provided the first evidence that CREB played a role in hematopoietic growth factor signal transduction in myeloid cells. CREB is also phosphorylated in response to thrombopoietin (TPO) in the Human Erythroleukemia (HEL) cell line (25). Additional studies showed that IL-3 stimulation results in CREB phosphorylation by a p38-MAP kinase-independent pathway (26–28).

CREB is a Ubiquitous Transcription Factor that Controls Cell Proliferation and Survival

CREB is a 43 kDa basic/leucine zipper (bZIP) transcription factor that is expressed in most tissues and is conserved from *Drosophila* to humans (Fig. 1). CREB binds to the octanucleotide cAMP response element (CRE) TGANNTCA as a homodimer and as heterodimers in conjunction with other members of the CREB/ATF superfamily of transcription factors (29, 30). The transcriptional function of CREB becomes activated after its phosphorylation on serine 133. Reflecting the fact that CREB can integrate signals from a variety of stimuli, this serine residue can be phosphorylated by a number of serine-threonine kinases. This includes the cAMP-dependent protein kinase A, calcium, calmodulin-dependent kinases (responding to calcium fluxes from either the extracellular environment or from internal calcium stores), or pp90 ribosomal S6 kinase, which is frequently activated downstream of growth factor receptors (Fig. 1). The phosphorylation of CREB on serine 133 induces its transcriptional activity by promoting its interaction with the 256 kDa coactivator protein CREB-binding protein (CBP) (ref. 31). CBP, which is employed as a cofactor by many transcription factors, stimulates transcription by modulating chromatin through histone acetylation as well as recruiting the factors necessary for RNA polymerization. CREB has been demonstrated to promote pleiotropic effects, including cell proliferation, differentiation, and survival of both neuronal and hematopoietic cells (29,30). CREB, like most transcription factors, can mediate distinct effects in different cell types or in response to disparate stimuli. This ability of CREB is likely dependent on cooperative effects with other contemporaneously activated transcription factors or signaling pathways or on chromatin alterations such as DNA methylation or histone modification.

CREB Activates Target Genes Downstream of GM-CSF Signaling

CREB target genes function in metabolism, transcription, cell-cycle control, cell survival, DNA repair, growth control, immune regulation, reproduction, signaling, transport, and cytoskeletal plasticity (29–31). The fact that CREB-binding sites have been identified in the promoters of a number of target genes involved in proliferation, differentiation, and survival, including *Bcl-2*, *Egr-1*, and *MAP kinase kinase*, suggests that CREB has pleiotropic effects on myeloid cells. This has been confirmed by studies to identify the CREB “regulon” (32).

CREB is a Critical Regulator of Myelopoiesis

In both mouse and humans, CREB is expressed more highly in less differentiated hematopoietic stem cells (HSCs), e.g., kit+sca+lin-, common myeloid progenitor (CMP), common granulocyte-macrophage progenitor (GMP), megakaryocyte-erythroid progenitor (MEP), multipotent progenitor (MPP) cells, compared to more committed cells (33). Knockdown of CREB in normal myeloid progenitor cells results in decreased myeloid proliferation in colony

assays and affects short-term engraftment. However, CREB downregulation does not have effects on long-term hematopoietic engraftment (33). Therefore, although CREB is important for the regulation of normal myelopoiesis, it does not appear to be necessary for hematopoietic reconstitution or definitive HSC activity.

Clinical-Translational Advances

CREB overexpression in AML patients

CREB has recently been demonstrated to be overexpressed in leukemic blast cells from patients with AML compared to normal bone marrow (34–36). Furthermore, CREB overexpression was associated with a worse prognosis in AML patients compared to patients whose bone marrow did not overexpress CREB (36–38). Overexpression of CREB protein did not correlate closely with CREB mRNA levels, suggesting that posttranscriptional mechanisms may contribute to the elevated expression. Furthermore, activation of CREB in leukemia cells appears to be cAMP independent (35,39). In all cases in which AML patient bone marrow samples overexpressed CREB, an increased level of phosphorylated CREB was also observed, indicating that the protein is functionally active (36). Analysis of primary AML blast cells by using fluorescence *in situ* hybridization revealed that there is an increase in copy number of the CREB gene (36). CREB overexpression also resulted in increased proliferation and survival of HSCs and myeloid cells *in vitro* and *in vivo* (36). Transgenic mice in which CREB is overexpressed in committed granulocyte and macrophage cells under the control of the hMRP8 promoter develop myeloproliferative disease (splenomegaly and aberrant myelopoiesis), but not AML, after a latency of 1 year (36). Bone marrow progenitor cells from these mice have increased replating activity and are hypersensitive to growth factors (36). Therefore, CREB plays a major role in the regulation of normal myeloid cell proliferation and differentiation and acts as a proto-oncogene that potentially contributes to leukemogenesis.

CREB knockdown in AML cells inhibits proliferation *in vitro* and *in vivo*

CREB downregulation in the AML cell lines TF-1 and K562 resulted in decreased proliferation in liquid culture with decreased cells in S phase. This appears, at least in part, to be due to a decrease in the expression of cyclins A1 and D. Both cyclins A and D regulate the G1-to-S transition and have been demonstrated to be CREB target genes (36). Transplantation of Ba/F3 pro-B cells transduced with a Bcr-Abl fusion protein containing the T315I mutation and CREB shRNAs or control scrambled shRNA into SCID mice resulted in delayed leukemia cell progression and prolonged survival (33). Given the fact that AML cells are more dependent on CREB than normal myeloid progenitor cells, CREB and CREB-dependent pathways may, therefore, be potential targets for drug development.

Molecular pathways downstream of CREB

Because CREB is primarily a transcriptional activator, the key to understanding how CREB regulates normal and aberrant myelopoiesis was to identify target genes downstream of CREB. One strategy by which to address this issue involved overexpressing CREB in a leukemic cell line and determining the resultant changes in gene expression (37). Among the most upregulated genes were *Meis1* and *Pbx1*, at 33-fold and 28-fold, respectively, compared to parental cells. Studies to determine whether *Meis1* and *Pbx1* are direct targets of CREB are currently in progress. Genes that were upregulated or downregulated in CREB knockdown cells were also analyzed (40). Among the most downregulated genes, interestingly, were histones. The decreased expression was validated by using RNA interference targeting CREB in myeloid leukemia cell lines and primary AML cells (40). The role of CREB in regulating histones is currently under investigation.

CREB in lung cancer

Altered CREB expression and activity play a role not only in leukemia, but in nonhematologic malignancies as well. Increased expression and phosphorylation of CREB has been found in non-small cell lung cancer cell lines, compared to nontransformed bronchial epithelial cell lines, and in pathologic samples from tumors compared to normal adjacent epithelium (41). That this finding is likely to be biologically important is suggested by the observation that patient survival was inversely related to CREB expression and phosphorylation. Interestingly, this prognostic effect of CREB was found in patients who had never smoked cigarettes rather than in current or former smokers.

Since CREB is regulated by several distinct pathways that can become activated in cancer cells, a key question concerns whether the activation of CREB seen in cancer cells is directly driving the malignant phenotype of the cells, or whether it is merely a byproduct of activation of one of these upstream pathways. This is a critical question, as CREB would represent a good molecular target only if it was playing a central role in the biology of the tumor. CREB activation has been found in non-small-cell lung cancer cell lines, displaying constitutively activated ERK and pp90RSK and increased expression of Bcl-2 and Bcl-xL (42). A dominant-negative mutant of CREB and siRNA to knockdown CREB inhibited the proliferation and survival of NSCLCs. Furthermore, an inhibitor of ERK and pp90RSK activation of CREB resulted in cell-cycle arrest at G2/M and increased apoptosis, suggesting that CREB is a potential target for therapy for NSCLC (42). Treatment of lung adenocarcinoma cells with these drugs resulted in decreased CREB phosphorylation, proliferation, and expression of cyclin D1 (43). CREB appears to upregulate specific target genes, such as *cox-2* in lung adenocarcinoma cells, through PKA, PKC, and ERK signaling pathways (44).

Therapeutic considerations of targeting transcription factors

Abundant evidence suggests that transcription factors like CREB play a key role in mediating the malignant behavior of tumor cells. Thus, the two key questions that arise are (1) can these proteins can be targeted effectively in patients, and (2) what are potential toxicities of therapies targeting transcription factors.

Pharmaceutical companies have a longstanding and active program in developing kinase inhibitors, nearly all of which focus on the same site, the ATP-binding domain. The structures of these pockets have been well characterized, and drug-like molecules with molecular weights of approximately 500 daltons can bind to these sites with high specificity. Transcription factors, by contrast, provide a more challenging target. The surfaces of interaction that they employ in protein-protein and protein-DNA interactions are somewhat larger. Furthermore, as with CREB, transcription factors are usually members of large families, and the likely toxicity of drugs targeting these proteins is much greater if multiple family members are affected.

However, notable advances have been made in the area of developmental therapeutics targeting transcription factors. Much of this advance has come from academic laboratories, perhaps reflecting the relative lack of interest of pharmaceutical and biotechnology companies in these proteins. Two types of strategies have been particularly fruitful. The first has been structure-based approaches in which inhibitors of specific domains are targeted. This has included inhibition of transcription factor phosphorylation, nuclear localization, DNA binding, and coactivator recruitment. The second approach has been to establish screening systems often relying on a transcription-based readout. For example, cell lines have been established in which the expression of reporter proteins such as luciferase, green fluorescent protein (GFP), or secreted alkaline phosphatase (SEAP) are under the control of a specific transcription factor. Large chemical libraries can then be screened for modulators of the pathway, while counterscreening against an unrelated pathway to exclude nonspecific compounds. This

approach, which has yielded compounds that can modulate a number of transcription factors (45,46), provides proof of concept that such specific inhibitors can be developed. In addition, this “chemical biology” approach provides great insight into mechanisms by which these pathways can be modulated therapeutically.

Conclusions

Starting with basic questions as to how gene expression in the nucleus is modulated by changes in the intracellular and extracellular environment, CREB has been identified as an important transcription factor in normal cellular function. The additional finding that CREB can mediate the effects of cytokines provided insight into how this protein plays a critical role in the homeostasis of complex systems such as hematopoiesis. This work then led to our understanding of the role that CREB can play in both hematologic and nonhematologic malignancies, and how it may be a target for rational therapy. This serves as an additional example of how basic advances can ultimately lead to an enhanced understanding of the pathogenesis of cancer, and new strategies for targeted rational therapy.

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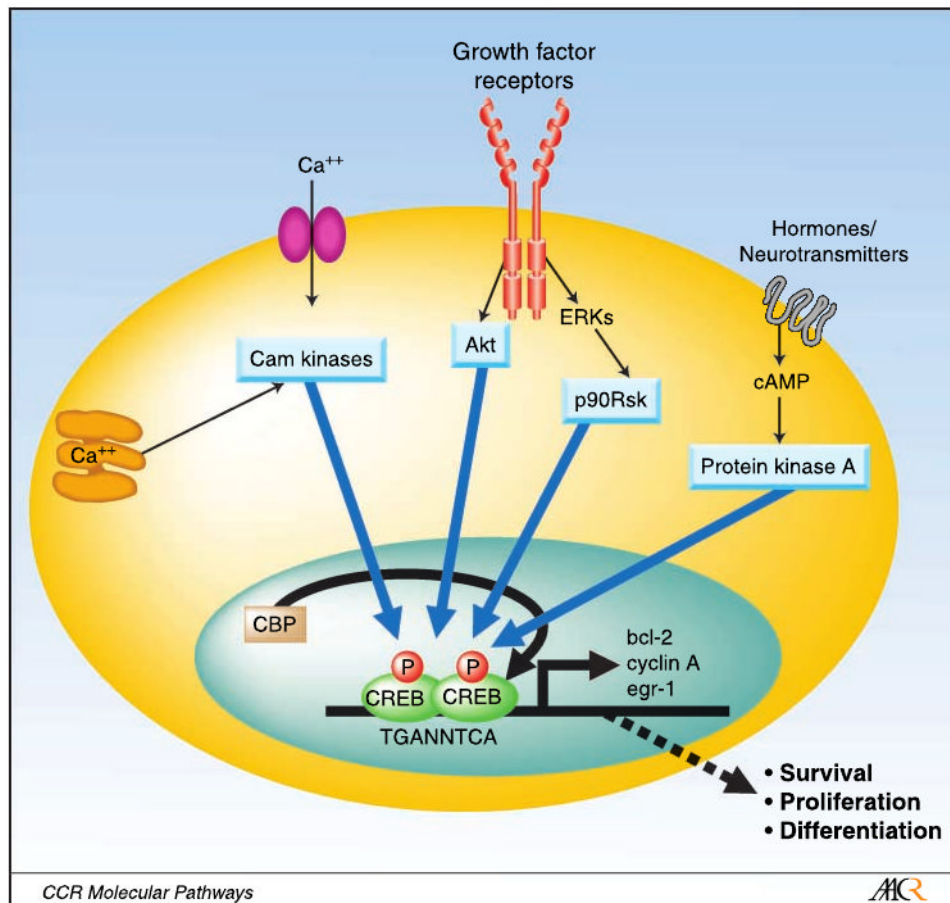
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**Fig. 1.**

CREB integrates signals from diverse cellular events to regulate the transcription of key target genes. The transcriptional function of CREB becomes activated when it is phosphorylated on serine 133, which triggers the recruitment of transcriptional coactivators such as CREB-binding protein (CBP). The phosphorylation of CREB can be catalyzed by a variety of kinases, including calcium/calmodulin-dependent (Cam) kinases, which can be activated by calcium fluxes from extracellular or intracellular compartments; Akt or p90Rsk, which can be activated by distinct pathways downstream of growth factor receptors; and protein kinase A, which is activated by cAMP. Genes regulated by CREB can control critical cellular processes, and thus the inappropriate activation of CREB can contribute to the pathogenesis of a variety of cancers.